Chapter

Random Mutagenesis of Filamentous Fungi Strains for High-Yield Production of Secondary Metabolites: The Role of Polyamines

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Abstract

A filamentous fungus (also called molds or moldy fungus) is a taxonomically diverse organism from phylum *Zygomycota* and *Ascomycota* with filamentous hyphae and has the ability to produce airborne spores or conidia. Currently, more than 70,000 molds are known, and some of them contain unique and unusual biochemical pathways. A number of products from such pathways, especially, the secondary metabolite (SM) pathways are used as important pharmaceuticals, including antibiotics, statins, and immunodepresants. Under different conditions, the individual species can produce more than 100 SM. The strain improvement programs lead to high yielding in target SM and significant reduction of spin-off products. The main tool for the strain improvement of filamentous fungi is random mutagenesis and screening. The majority of industrial overproducing SM strains were developed with the help of such technique over the past 50–70 years; the yield of the target SM increased by 100- to 1000-fold or more. Moreover, most of the strains have reached their technological limit of improvement. A new round of mutagenesis has not increased overproduction. Recently, it was shown that that the addition of exogenous polyamines may increase the production of such improved strains of filamentous fungi. The possible molecular mechanism of this phenomenon and its biotechnological applications are discussed.

Keywords: filamentous fungi, random mutagenesis and screening, strain improvement, secondary metabolites, polyamines

1. Introduction

Improved strains of filamentous fungi are widely used in the biotechnology industry for recycling of secondary raw materials [1–3] as biosorbents [4], in fermentation of cheese [5], wine [6, 7], and other food products [8, 9], as well as for the production of enzymes [10–13], organic acids [14, 15], secondary metabolites (SMs) [16, 17], or for steroid transformation [18, 19]. There are four main tools for fungal strain improvement: (1) sexual crossing [20, 21], (2) somatic crossing (including parasexual recombination [22]), (3) random mutagenesis by physical or/and chemical

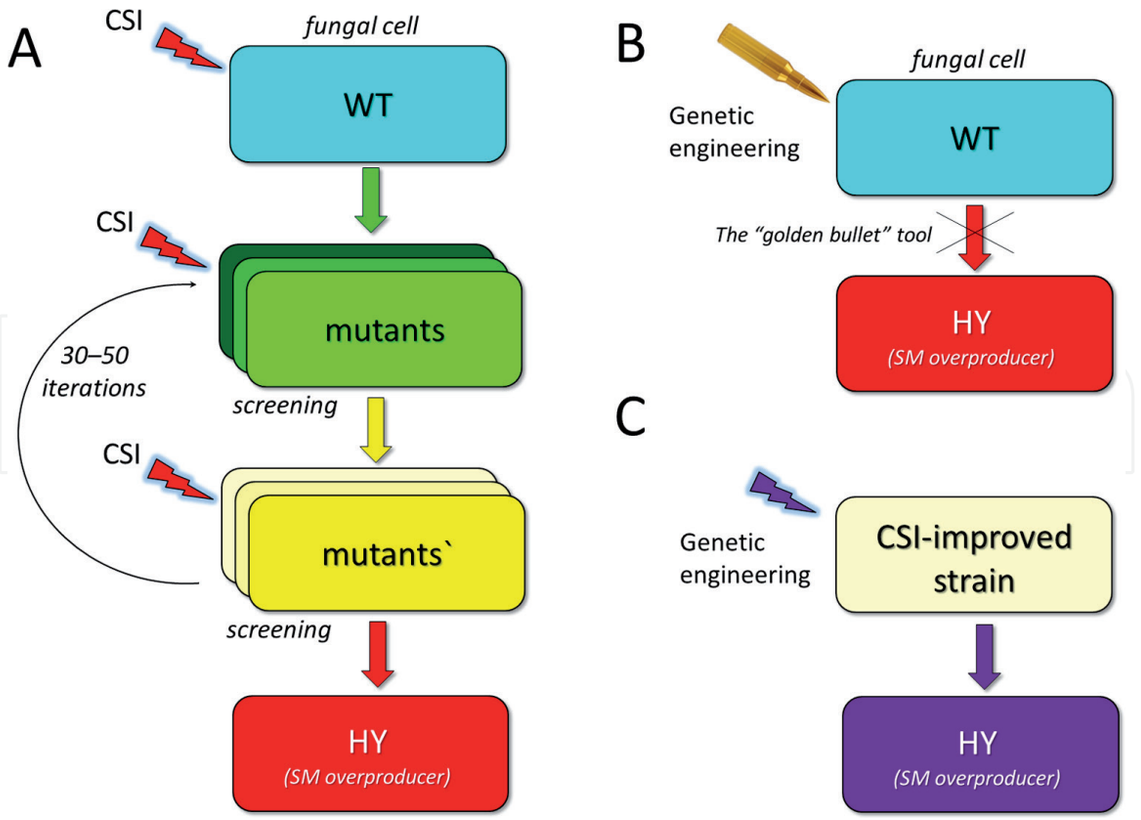

Figure 1.
The improving of filamentous fungi strains for SM production. (A) CSI programs for fungal strain improvement. (B) The WT strain improvement by the "golden bullet" tool from genetic engineering. (C) The combination of SCI and genetic engineering approaches for the developing of novel HY strain. SM: secondary metabolite, CSI: classical strain improvement, WT: wild type, and HY: high yielding.

mutagens and screening [23, 24], and (4) genetic engineering [25–27]. These methods can be applied separately or in various combinations [28]. The first three tools are referred to as classical strain improvement (CSI) methods and have been used in strain improvement programs for filamentous fungi for SM production since the 1950s of the twentieth century (**Figure 1A**). The majority of industrial producers of secondary metabolites in fungi were obtained precisely with the use of CSI [25]. The powerful genetic engineering approach has been available since the end of the twentieth century for targeting the particular genetic determinant to introduce novel properties into an organism [28]. Since the improvement of filamentous fungi strains for SM production is a complex and multistage program that radically changes numerous processes, there is no "golden bullet," any single unique genetic change to produce high yielding (HY) strain from the wild type (WT) strain [29] (**Figure 1B**). However, introducing of novel targeted features into already improved strains enables to create SM-overproducing strains [30]. For instance, the introduction of the compactin pathway from the *Penicillium citrinum*, as well as CYP105AS1 (from *Amycolatopsis orientalis*, for pravastatin hydroxylation) into the β-lactam-negative *P. chrysogenum* DS50662 strain, yielded more than 6 g/L of pravastatin [25]. This seems to be due to the interaction of different tools that are available to improve strains (**Figure 1C**).

2. CSI for SM production in filamentous fungi

For the majority of industrially important filamentous fungi (except members of genera *Aspergillus*, *Claviceps*, and *Emericellopsis*), the sexual breeding is not available [28]. From the other side, a number of these organisms produce haploid

conidia, which provide the ideal material for mutagenic treatment: in the absence of a complementary set of genes, mutations will be easily detected using suitable screening and the stability of the mutant will generally be good [28]. Filamentous fungi turned out to be surprisingly tolerant to strong mutagenic effects, retaining their strength and productivity even after radical rearrangements of their chromosomes [31, 32]. In this case, the main tool of CSI for SM production in filamentous fungi is random mutagenesis mutagens and screening [33].

2.1 The overproduction of target SM in filamentous fungi

The individual species of filamentous fungi under different external and internal signals are able to produce up to 100–150 or more different SMs [34–36]. This is achieved due to the presence in the genomes of these organisms of 30–80 clusters of genes, responsible for various biosynthetic pathways of SMs, so-called biosynthetic gene clusters (BGCs) [37, 38], and by the fine tuning in the regulation of their expression [39, 40]. Currently, more than 20,000 SMs are known to be produced from more than 1000 characterized gene clusters of filamentous fungi [35, 36]. Normally, gene clusters are "silent," the expression level of BGCs is extremely low, and there is practically no biosynthesis of any SMs (**Figure 2A**). For the biosynthesis of particular SM, the corresponding BGC must be "awakened" by some specific signal. For instance, the environmental signal 1 is resulted in the biosynthesis of SM1 (**Figure 2A**). A number of possible SMs, which can be produced in a particular organism after the "awakening" of corresponding BGCs, constitute its biosynthetic capacity for SMs (**Figure 2A**). In order to become an industrial producer, the fungus strain must increase the production the target SM 100- to 1000-fold or more (**Figure 2B**). It is also necessary that under favorable conditions (usually, these are fermentation conditions), the content of spin-off products would be extremely small (**Figure 2B**).

2.2 The molecular mechanisms of SM overproduction in filamentous fungi

An increase in the production of the target SM by 100- to 1000-fold and the elimination of spin-off products under the fermentation conditions in the improved fungal strains (**Figure 2B**) are associated with two main molecular events, the upregulation of genes from target BGC and the knockout of genes from alternative BGC [27, 33, 41, 42]. Since the expression of BGC genes is controlled by the pathway-specific regulation [27, 43, 44], global regulation [45, 46], and global regulation of SM [47–50], the SCI programs are accompanied by changes in such controls. For instance, during CGI program for penicillin G (PenG) production in HY strain (DS17690) two main events occurred, the shift in global regulation of secondary metabolism by introducing mutations in LaeA and VelA and mutations in key enzymes for spin-off SM [33]. That enabled to escape control from the global regulation of SM and involve more than one gene copy of BGC for PenG.

Usually, an increase in the gene dose (introducing several BGC copies for target SM) does not lead to an increase in gene expression. For instance, in the another PenG-overproducing strain (P2niaD18) the enhanced penicillin titer does not strictly depend on the copy number of the cluster [51]. This phenomenon occurs due to the control from the global regulation of SM, which brings only one cluster to work, the rest are silent [35, 52, 53]. Since there are 8 BGC copies for PenG in DS17690 strain, the escape of global regulation resulted in the significant increase in the yield of the target SM [33]. However, the shift in global regulation of SM could also significantly upregulate the expression from alternative BGC [54, 55]. From this point of view, it becomes clear why the CSI program for DS17690 strain

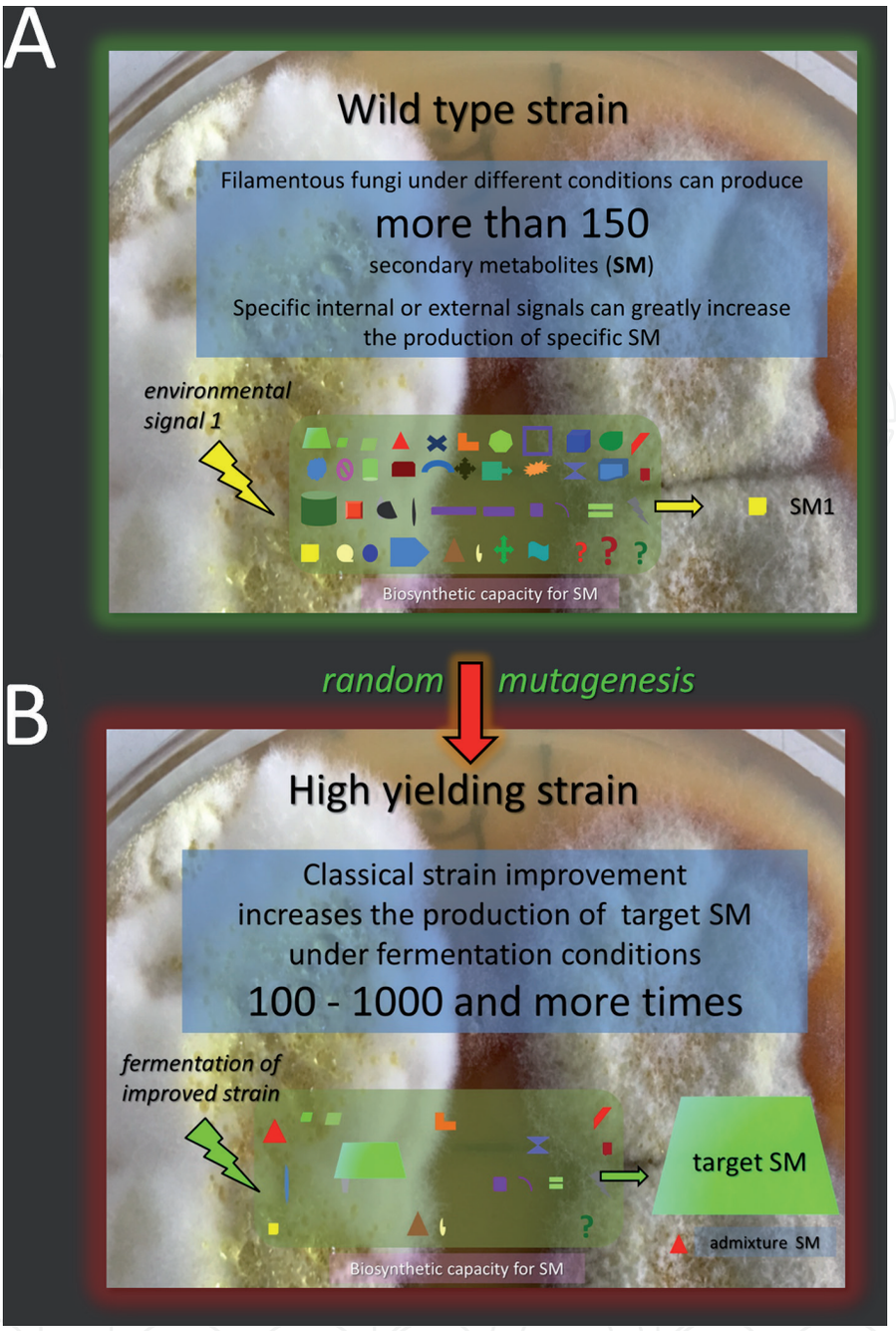

Figure 2.
The shift in biosynthetic capacity for SM production after CSI programs of filamentous fungi. (A) The response of WT strain to the environmental signal 1 and production of SM1. (B) The overproduction of target SM in the HY strain under fermentation conditions. SM: secondary metabolite, CSI: classical strain improvement, WT: wild type, and HY: high yielding.

changes in global regulation of SM also accompanied by mutations in central enzymes for spin-off SM [33]. The shift in global regulation of SM not only took out of control additional BGC copies for PenG, but also – BGC for spin-off SM. Since the screening went against spin-off SM, variants with mutations in central enzymes of alternative BGC were selected [33]. It was demonstrated, that delections in such central enzymes (NRPS or PKS megasynthases) lead by a still unknown mechanism to the silencing of all genes from the corresponding gene cluster [56].

Thus, if the improving of filamentous fungi strains for SM production led to duplication of target BGC, the simultaneous changes both in the system of global regulation of SM and at the level of alternative BGC expression are required [33]. However, in many improved strains, industrial producers of SMs, spin-off products are still formed during fermentation [57, 58]. These impurities are intermediates of the

target SM biosynthesis; their amount depends on numerous fermentation conditions [58]. For instance, the cephalosporin C (CPC) yield after fermentation of improved *Acremonium chrysogenum* strains often contaminated with deacetylcephalosporin C (DAC) [57]. DAC is immediate precursor of CPC in the biosynthetic pathway. The conversion from DAC to CPC is catalyzed by deacetylcephalosporin-C acetyltransferase enzyme (CefG; EC 2.3.1.175) by, occurs in the cytoplasm [59] and is utilizes one molecule of cytoplasmic acetyl-CoA per reaction. In HY strains the CPC production increased 200- to 300-fold and the expression from BGC for CPC (*cef* genes) upregulated 20- to 300-fold [41]. In this case the acetyl-CoA content may be depleted in some HY strains [57, 58]. From the other side the screening during CSI programs went the same way against DAC admixture, events that reduce CPC/DAC ratio were selected. In the *A. chrysogenum* HY strain RNCM 408D [60], the CPC/DAC does not exceed 10–15% [61]. Thus, to increase in SM production is accompanied not only by changes in the expression and regulation of BGCs, but by reprogramming the whole organism, starting with changes in the primary metabolism (for the needs of target SM biosynthesis), ending with changes in the transport and assimilation of nutrients, the ability to assimilate oxygen, adaptation to fermentation conditions, and much more [42]. That is why the improvement of the filamentous fungi strain is a multi-step process, involving alterations in many spheres of the strain's vital activity, and there is no "golden bullet," no one cardinal event that converts WT strain to HY (**Figure 1**).

2.3 The technological limit of CSI of filamentous fungi for SM production

Filamentous fungi are a good facility for the improving of SM production by random mutagenesis and screening [2, 28, 62, 63]. Among the most popular mutagens used for fungal strain improvement are DNA alkylating NTG (N′-methyl-N′-nitro-N′-nitrosoguanidine) which typically produces a variety of point mutations and UV irradiation at 254 nm, which causes the formation of pyrimide dimers leading to point mutations and deletions [28]. In general, the CSI program for SM production in filamentous fungi looks as shown in **Figure 3**. The WT strain produce target SM in most cases at a low level, usually it does not exceed 30–50 μg/ml of fermentation medium [27, 41]. On order to convert WT to HY strain a number of independent events, involving BGCs regulation, changes in primary metabolism, strain physiology and so on, must occur. Moreover, all these events do not have to happen simultaneously. There are a number of ways in which the production of target SM gains added benefit. The first round of mutagenesis against WT strain results in a series of mutants, some of them have shift in the production of target SM (**Figure 3**). The majority of alterations lead to decrease or lack of the production change, but some mutants may show the increase in SM production. They are used as origins for the next mutagenesis round, followed by the next stage of screening. For example, on the A′ round of mutagenesis, the production level of SM was increased by A%, on the B′ round of mutagenesis, the production level of SM was increased by B% (**Figure 3**). Thus the CSI gradually leads to the emergence of a whole set of changes leading to an increase in the production. However, along with beneficial changes that increase the production of the target SM, reduce the amount of spin-off products, and others, numerous side changes begin to accumulate in the fungal strain. They can appear in a slow growth on agar and liquid media [27, 64, 65], a decrease in stress resistance [66], reduction in the conidia formation [64] and many other properties, expressed in a decrease in the overall viability of the strain [66, 67]. Finally, the stage comes when the next mutagenic effect no longer leads to further strain improvement. This is the technological limit of CSI method, it comes for each improvement program for a particular strain of filamentous fungus and is usually found at the 10–50th round of mutagenesis [60, 68].

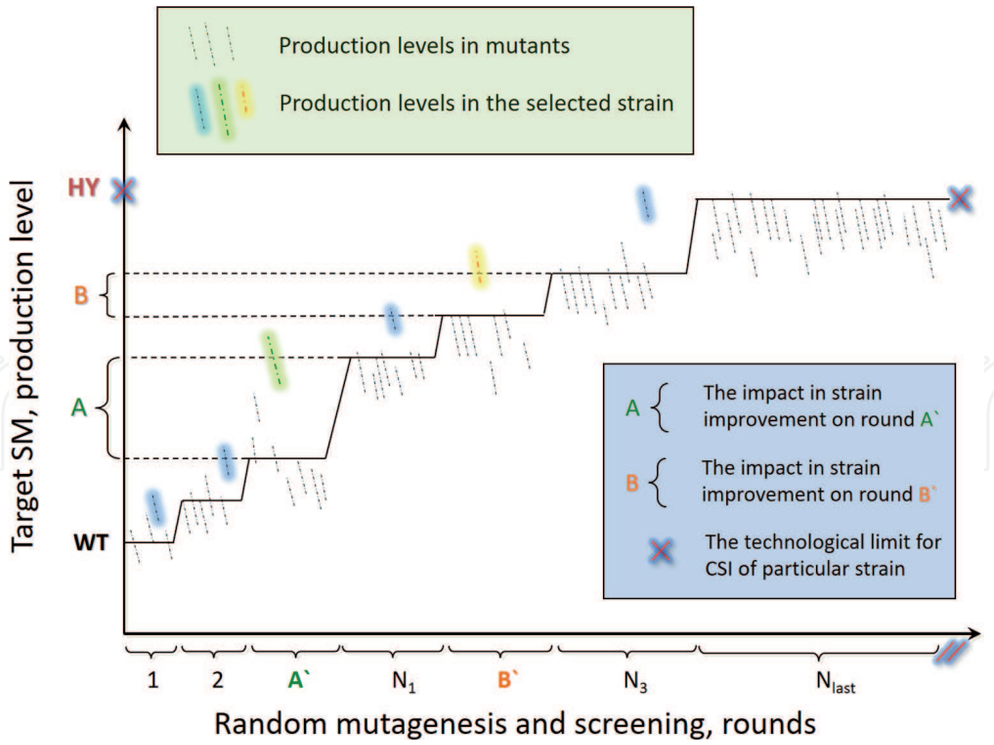

Figure 3.
Random mutagenesis and screening for the improving of filamentous fungi strains for SM production. SM: secondary metabolite, CSI: classical strain improvement, WT: wild type, and HY: high yielding.

3. Role of polyamines in filamentous fungi HY strains (after SCI for SM production)

Aliphatic polyamines (PAs) such as putrescine, spermidine, and spermine are widespread in nature; they are present in all living organisms and are also present in viral particles [69]. Despite the fact that these compounds have long been known as components of biological systems, there is still no clear understanding of their role in various bioprocesses [70]. The most studied functions of PAs are associated with stimulating the growth of microorganisms, increasing membrane stability, interacting with nucleic acids, and regulating the level of heterochromatin in the cell [71–74]. The roles of PAs in fungi cell have also been discussed [71, 75]. The main topics correspond to stress resistance [76], phytopathogenicity [77] and fungal development, including sporulation, growth and other stages of lifecycle [78, 79]. There is tight control of polyamine homeostasis in the cell [80]. For a particular organism, there is a certain content of PAs. For this, there are both biosynthetic and catabolic enzymes of polyamines (**Figure 4**), moreover, the amount of key biosynthetic enzymes, such as ornithine decarboxylase (ODC), or S-adenosylmethionine decarboxylase (AdoMetDC) is regulated at the levels of transcription, translation and turnover rate (half-life) [80, 81].

3.1 Influence of PAs on SM production in improved strains

Recently it was demonstrated, aliphatic PAs, such as 1,3-Diaminopropane (DAP) or spermidine (Spd) may increase the production of target SMs in HY fungi strains [82]. The production level of PenG in *P. chrysogenum* increased by 10–15% [82], the CPC production in *A. chrysogenum* HY strain increased by 10–15% [83] and the production of lovastatin (LOV) by *Aspergillus terreus* HY strain at the particular timepoints of fermentation increased by 20–45% [84]. The addition of 5 mM PAs

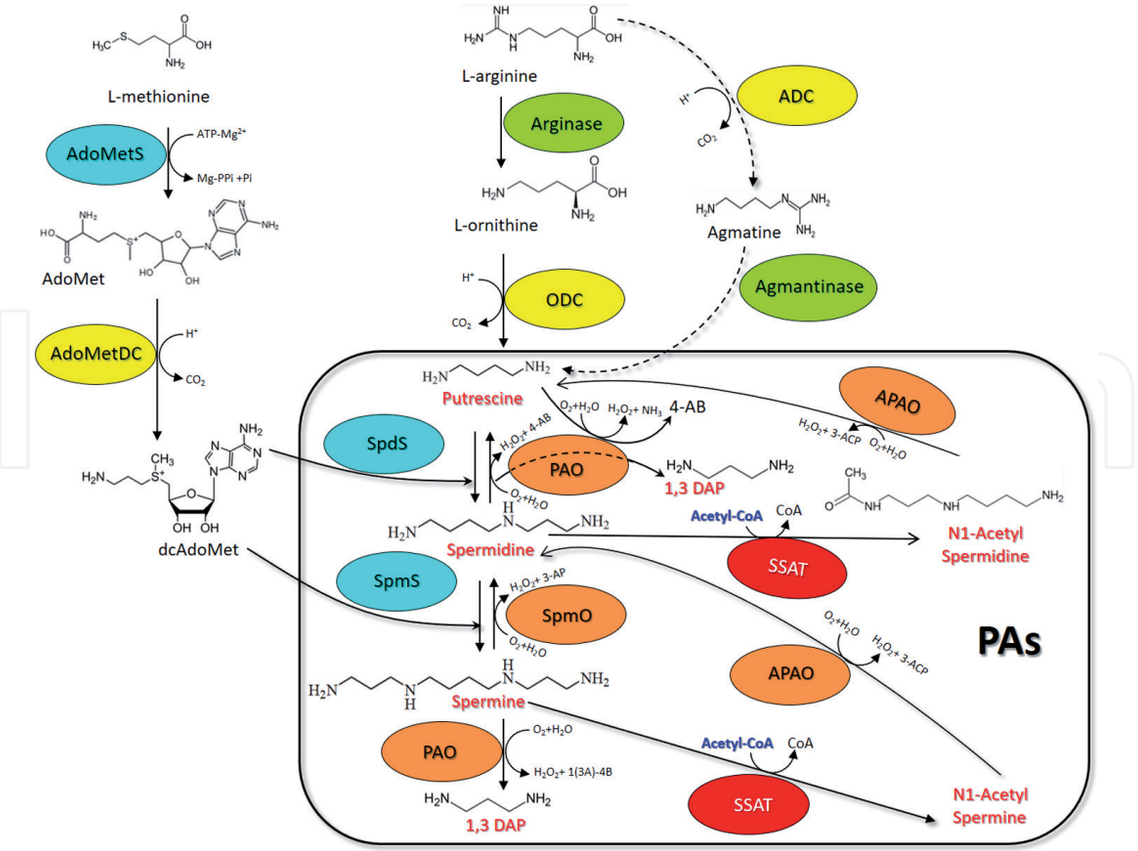

Figure 4.
The metabolism of polyamines in filamentous fungi. PAs: polyamines, ODC: ornithine decarboxylase, ADC: arginine decarboxylase, AdoMetS: S-adenosylmethionine synthetase, AdoMetDC: S-adenosylmethionine decarboxylase, SpdS: spermidine synthase, SpmS: spermine synthase, PAO: polyamine oxidase, SpmO: spermine oxidase, SSAT: spermidine/spermine-N1-acetyltransferase, APAO: N-acetylpolyamine oxidase, DFMO: α-difluoromethylornithine, APA: 1-aminooxy-3-aminopropane, DFMA: α-difluoromethylarginine, and AO-Agm: 1-aminooxy-3-guanidinopropane.

to agar media increased the survival of HY strains, as demonstrated by the drop and dilution assay [83, 84]. The PAs addition during the fermentation of improved strains also led to upregulation of corresponding BGCs (*pen*, *cef* and *lov* genes) [82–84]. This is important because *A. chrysogenum* and *A. terreus* HY strains have reached their technological limit after CSI programs [60, 68] and the possibility of further increasing the production of valuable pharmacologically significant substances as a result of the addition of relatively cheap PAs may be significant in the biotechnology industry.

3.2 Possible mechanisms of influencing of exogenous PAs on SM production

The addition of exogenous PAs also accompanied by the *laeA* upregulation in all studied improved strains [82–84]. LaeA is global regulator of SM in filamentous fungi [50]. It is S-adenosylmethionine (SAMe)-dependent histone methylate, which acts epigenetically, through the chromatin remodeling [85]. Since the biosynthesis of PAs and the work of LaeA require the same substrate, SAMe, the addition of exogenous PAs can lead to a shift in the global regulation of the studied HY strains. It is also known that in all these strains, *P. chrysogenum* Wis 54-1255, *A. chrysogenum* RNCM 408D and *A. terreus* No. 44-62, the only one copy of corresponding BGC is present, one copy of *pen* genes [86], one copy of "early" and "late" *cef* genes [32, 41] and one copy on *lov* genes [84] respectively. In this regard, the CSI programs for these strains could follow a rather different pathway than *P. chrysogenum* DS17690

with eight copies of *pen* genes [30], without significant shifting and removing the global regulation of SM and mutation in LaeA.

In order to confirm this hypothesis we carried out fermentation with PAs for WT, HY and E6 strains of *A. terreus* [44]. The LOV production in *A. terreus* is under the control of two major positive regulators, the LovE pathway-specific regulator and LaeA global regulator of fungi SM [44]. The *A. terreus* E6 strain derived from WT by the genetic engineering introduction the additional copy of *lovE* gene under the control of constitutive promotor [27]. LovE is Zn_2Cys_6 transcription factor for pathway-specific regulation of *lov* genes; in *A. terreus OE::lovE* the LOV production increased 10- to 12-fold [27]. Surprisingly, the addition of PAs during the fermentation of the E6 strain led, on the contrary, to a decrease in LOV production (**Figure 5**). But it is also known that pathway-specific regulators can negatively regulate LaeA [49]. For instance, AflR, a sterigmatocystin pathway-specific transcription factor, negatively regulate the expression of *laeA* [49]. E6 strain has the only one targeted change in the genome of WT, that led to constitutive (which also means LaeA-independent) overexpression of *lovE*. The effects of *laeA* downregulation (due to an increase in the dose of the negative regulator gene) on LOV production in E6 strain are compensated by *lovE* upregulation [49]. However, LovE, unlike LaeA, upregulate only *lov* genes for biosynthesis, not for transport and resistance Therefore, the PAs addition during fermentation of E6 strain causes a toxic effect and the LOV production decreases (**Figure 5**).

3.3 The endogenous polyamines content in *A. chrysogenum* HY strain

Since exogenous PAs are able to influence the production of SM in HY strains, it is important to know if there have been any changes in the metabolism and homeostasis of polyamines into the cells of improved fungi strains. Recently it was demonstrated, that *A. chrysogenum* HY strain shows increased resistance to inhibitors

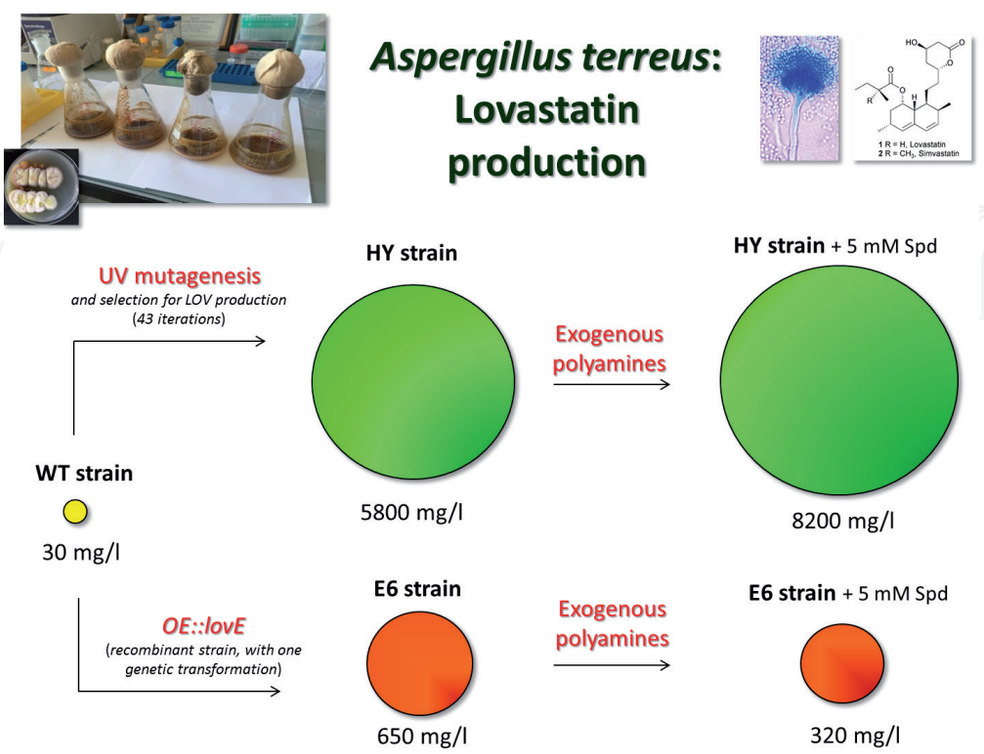

Figure 5.
Effect of exogenous polyamines on lovastatin production in the WT, E6, and HY A. terreus *strains. WT: wild type, E6:* OE::lovE*, HY: high yielding, and Spd: spermidine. Adapted with permission from [44].*

of ODC, the key enzyme of PAs biosynthesis, on minimal agar media [65]. The addition of 5 mM α-difluoromethylornithine (DFMO) or 5 mM of 1-aminooxy-3-aminopropane (APA) completely inhibited the growth of the WT strain, unlike HY strain (**Figure 4**) [65]. Such kind of resistance against inhibiters of key enzyme for PAs production turned out to be rather strange since HY strain is significantly weakened after SCI program [32, 61, 64, 66, 67]. The only previously observed advantage over the WT strain was expressed in CPC overproduction [41]. To explain the phenomenon of the resistance of HY strain to ODC inhibitors, an inhibitory analysis of *A. chrysogenum* WT and HY strains was performed against all pathways of putrescine biosynthesis (**Figure 3**). In filamentous fungi, in addition to the main pathway of putrescine (Put) biosynthesis, via ODC, there is also an additional pathway through arginine decarboxylase (ADC) and biosynthesis of agmatine (**Figure 3**). The inhibitory analysis demonstrated shift from the ADC-dependent to ODC-dependent biosynthesis of Put. During the fermentation for CPC production the total PAs content into HY strain has been increased by about fivefold [65].

The reasons for the increased production of PAs in the HY strain were discussed [65]. One on the reasons may be related to strain improvement techniques. The increasing in PAs content may be spin-o result of mutagenesis and DNA damage. Recently it was demonstrated that PAs can maintain the genome integrity via homology-directed DNA repair, enhancing the DNA strand exchange activity of RAD51 recombinase [87]. PAs also can protect DNA from free-radical damage by reacting direct with the reactive oxygen species [88–90].

4. Conclusions

As a result of CSI programs for filamentous fungi, a number of pharmaceutically significant SMs have been overproduced. One of the side effects of the high yielding strains improvement may be an increase in the content of polyamines (PAs). An increase in the PAs' content could occur as a response to mutagenesis during CSI. The recently discovered increase in the production of targeted SM in some HY strains after the addition of exogenous PAs may occur due to a decrease in endogenous biosynthesis of PAs and the release of additional resources for the biosynthesis of the target SM.

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Conflict of interest

The author declares no conflict of interest.

Notes/thanks/other declarations

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